several items are present in the visual field may be a result of this limitation[16,17]. The asymmetry in attentional resolution between the upper and lower visual field may also contribute to the reported lower visual field advantage for the perception of global shape in tasks involving hierarchical structures where several elements are used[18–20].

We attempted to locate the cortical area that mediates visual attention and limits the final access to our conscious vision. We show that stimuli not available to conscious perception could produce undiminished orientation-specific adaptation, a process that first occurs in primary visual cortex. Clearly, neuronal activity in the primary visual cortex is not sufficient for conscious perception. This is further supported by the fact that although there is little anatomical asymmetry between the upper and lower visual fields in human primary visual cortex[10–12], crowding and other manipulations of attentional load produced a large asymmetry favouring the lower visual field. The same holds for V2 as well. The dorsal parietal system has classically been associated with attentional processes[21,22] and the projections from early visual areas to the parietal regions are more numerous for the lower visual field than the upper field[23]. We suggest that the dorsal parietal area may control attentional resolution and the information entering our conscious vision. □

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Homodimeric architecture of a ClC-type chloride ion channel

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THE recent discovery of the ClC-family of anion-conducting channel proteins[1–3] has led to an appreciation of the central roles played by chloride ion channels in cellular functions, such as electrical behaviour of muscle[4–7] and nerve[8] and epithelial solute transport[9]. Little is known, however, about molecular architecture or sequence–function relationships in these membrane proteins. In the single case of ClC-0, a voltage-gated 'muscle-type' chloride channel, the functional complex is known to be a homo-oligomer of a polypeptide of $M_r \sim 90{,}000$, with no associated 'helper' subunits[10]. The subunit stoichiometry of ClC-type channels is controversial, however, with either dimeric or tetrameric association suggested by different indirect experiments[10,11]. Before a coherent molecular view of this new class of ion channels can emerge, the fundamental question of subunit composition must first be settled. We have examined hybrid ClC-0 channels constructed from functionally tagged subunits, and report here that ClC-0 is a homodimer containing two chloride-conduction pores.

These experiments examine ClC-0 channels purified and functionally reconstituted after high-level heterologous expression in mammalian cells. The expressed channels display the same molecular and functional characteristics observed[10] with ClC-0 purified from its natural source, *Torpedo* electroplax (Fig. 1). Both preparations run identically on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) gels as single, lightly glycosylated 90K bands; the expressed, purified channels incorporated into planar lipid bilayers show the same single-channel conductance and voltage-dependent gating behaviour as the native *Torpedo* channel[12–14]. An unusual characteristic of ClC-0 channels—and one essential to grasp for addressing questions of molecular structure—is the existence of two identical and physically separate Cl^--conduction pores in a 'double-barrelled' complex[12–16]. This feature is manifested in the single-channel records by three distinct, equally spaced conductance levels, in which the pores are either both closed (level 0), one open and one closed (level 1), or both open (level 2). Because the two pores are identical, level 1 is degenerate, the conductance being the same regardless of which pore is open.

Recently, residue K519, putatively located on the cytoplasmic side of the membrane just after the twelfth transmembrane domain, was found by macroscopic electrophysiological experiments to influence gating and anion permeation[17]. The single-pore conductance is sensitive to the charge at this position (Fig. 2). Similar conductances are seen for K519 (16.1 ± 1 pS) and R519 (13.1 ± 0.3 pS), whereas electrically neutral substitutions, regardless of their chemical nature (C519, Q519, M519, F519), yield channels with about half this conductance (5–7 pS). Substitution of a negative residue lowers the conductance further (2–3 pS). The dependence of conductance on side-chain charge argues that the residue influences Cl^- permeation primarily by an electrostatic mechanism. A small but statistically significant effect of side-chain volume may also operate because the R519 conductance is lower than that of K519 and the F519 conductance falls below those of the smaller neutral replacements. All these charge-substituted channels display the three binomially distributed gating levels of the double-barrelled channel complex[12,13], in which level 2 has precisely twice the conductance of level 1. The preferential effect of charge substitutions on inward current (Fig. 2*b* and ref. 17) indicates that residue 519 might be located on the cytoplasmic side of the channel, a weak suggestion that we unambiguously confirm below.

The single-channel conductance can be used as a functional tag in subunit-mixing experiments to deduce the number of ClC-0-519 residues contributing to the pore, and hence the number of subunits in the homo-oligomer. We coexpressed complementary DNAs coding for K519 and C519, purified the resulting channels, and examined the single-channel permeation properties of these

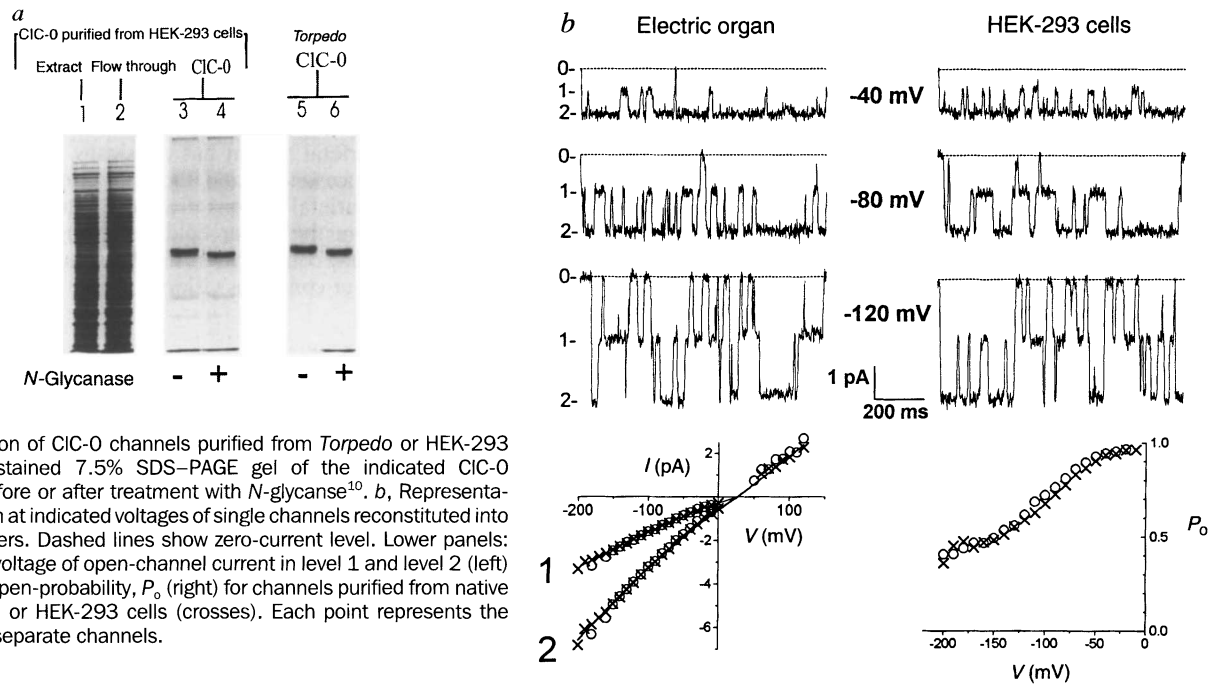

FIG. 1 Comparison of ClC-0 channels purified from *Torpedo* or HEK-293 cells. *a*, Silver-stained 7.5% SDS–PAGE gel of the indicated ClC-0 preparations, before or after treatment with *N*-glycanse[10]. *b*, Representative records taken at indicated voltages of single channels reconstituted into planar lipid bilayers. Dashed lines show zero-current level. Lower panels: dependence on voltage of open-channel current in level 1 and level 2 (left) or steady-state open-probability, P_o (right) for channels purified from native *Torpedo* (circles) or HEK-293 cells (crosses). Each point represents the average of 3–5 separate channels.

hybrids. These experiments optimized yields of hybrid channels by exploiting the opportunities offered by the immunoaffinity purification procedure. In K519, the natural 'RM2' epitope[10] was replaced with a haemagglutinin (HA) epitope, a manoeuvre that did not affect channel function (data not shown), whereas in C519 channels the RM2 epitope was preserved. In a coexpressed mixture, we could thus purify only C519 homo-oligomers and C519–K519 hetero-oligomers, while discarding the wild-type K519 homo-oligomers, as illustrated in Fig. 3*a*. Western blots (not shown) confirm that the purified ClC-0 preparations carry both the RM2 and HA epitopes.

Upon reconstituting these preparations into planar lipid bilayers, we observed two types of single ClC-0 channels. Of 17 channels observed, four were indistinguishable in all respects from homomeric C519, and one behaved like a wild-type K519 homomer contaminating the preparation. In contrast, 12 displayed a striking hybrid behaviour never before observed in ClC channels. Rather than showing three conductance levels, these hybrid channels exhibit four, labelled 0, 1C, 1K, and 2 in Fig. 3*b*. Level 0 is nonconducting, as with homomeric ClC-0. The intermediate states 1C and 1K have precisely the single-pore conductances of the corresponding homo-oligomeric channels C519 and K519, as is clear from the aligned amplitude histograms (Fig. 3*b*) and from the summary (Fig. 3*c*) of our compiled observations from three coexpressed channel preparations. Moreover, the level 2 conductance is the sum of the level 1C and 1K conductances, a result that provides additional evidence that these gating substates indeed represent two physically distinct Cl^--conduction pores in parallel. The degeneracy of homomeric level 1 is broken in the hybrid channel, because now the two pores have different conductances. Thus, the hybrid ClC-0 complex viewed as a whole displays a qualitatively novel behaviour not seen in either parental form; each individual pore, however, carries the pure conductance phenotype of its parent. Figure 3*c* also indicates that this was the only hybrid phenotype observed. We did not discern any intermediate pore-conductances, as with the multiple hybrid

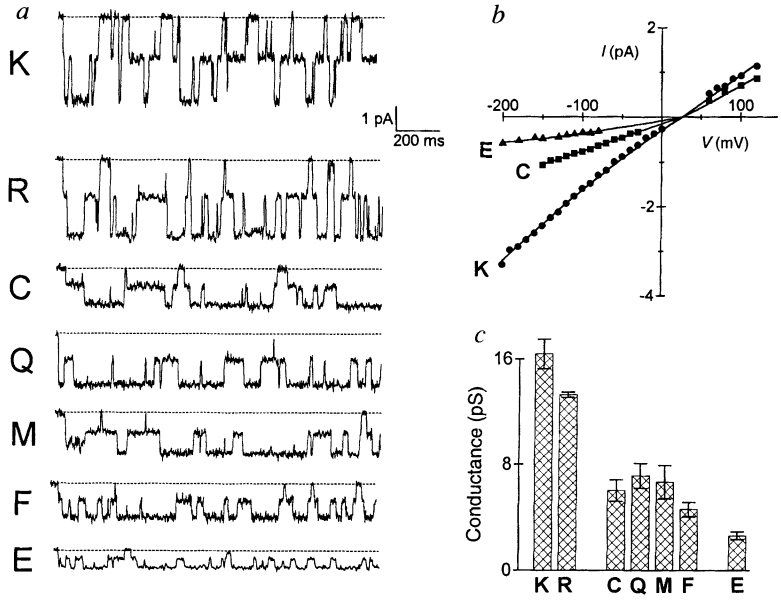

FIG. 2 Electrostatic charge substitutions at residue 519. ClC-0 channels with the indicated residues at position 519 were expressed in HEK-293 cells, purified and reconstituted into planar lipid bilayers. *a*, Single-channel recordings at −120 mV are shown for each ClC-0 variant. *b*, Single-pore *I*–*V* curves are shown for K519 (circles), C519 (squares) and E519 (triangles). *c*, Single-pore slope conductance (in the range −100 to −150 mV) of ClC-0 variants. Bars represent the means ±s.d. of 2–9 determinations on separate channels. ClC-0 mutants were constructed by PCR-based cassette mutagenesis and verified by sequencing.

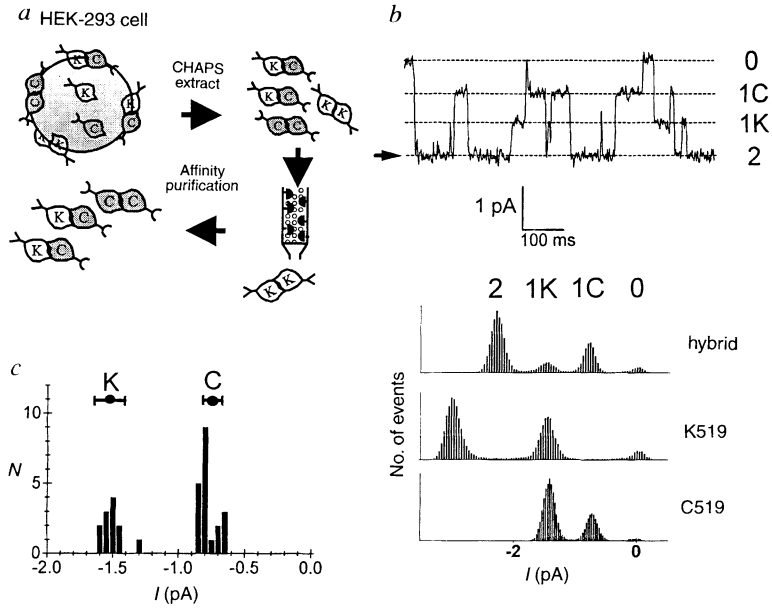

FIG. 3 K519–C519 hybrid ClC-0 channels. *a*, Purification scheme selecting for channels containing C519 subunits. *b*, Upper panel: recording at −100 mV of a K519–C519 hybrid, showing the four conductance levels (dashed lines); arrow marks the sum of the 1C and 1K currents. Lower panel: amplitude histograms for parental (C519 or K519) and hybrid channels. *c*, A histogram of a single-pore current at −100 mV was constructed from a total of 32 pores observed in 3 preparations of coexpressed K519–C519. Filled points with horizontal standard deviation bars locate the mean currents of 9–11 homo-oligomeric K519 or C519 channels at this voltage.

pores that arise in coexpressed subunit mixtures of pentameric acetylcholine receptors[18] or tetrameric cyclic nucleotide-gated channels[19]. Therefore, the 519 residue manifests its influence only once in each pore.

We carried out additional subunit-counting experiments with homo-oligomeric C519 channels. The approach exploits the chemical reactivity of cysteine with methanethiosulphonate reagents[20,21] and the electrostatic influence of residue 519 on single-channel conductance. As outlined in Fig. 4*a*, we ask how many cysteine-modification events are required to convert the low-conductance C519 channel to the high-conductance phenotype expected from reaction with methanethiosulphonate ethylamine (MTSEA), which produces a positively charged side chain. We find that 1 μM MTSEA added to the cytoplasmic side of C519 channels fully reverts the mutant channel to a high-conductance phenotype in a two-step reaction within 1–2 min (Fig. 4*b*). At the single-channel level, each of the two steps is individually observable. The first step (middle trace) converts one of the 6pS C519 pores to 13 pS, without affecting the other pore; following this first 'hit' by MTSEA, the channel displays four-level gating reminiscent of the coexpressed C519–K519 hybrids described above. About 30 s later, the remaining C519 pore is converted to a high-conductance pore by a second MTSEA hit (lower trace), and the channel again gates among only three levels. These conversion events were observed in all 19 attempts to react ClC-0–C519 channels with MTSEA.

Other charged MTS reagents alter the C519 pore as well (Fig. 4*c*). MTSET, a membrane-impermeant quaternary ammonium analogue of MTSEA, converts the C519 channel to a high-conductance phenotype in two discrete steps, but only when added to the cytoplasmic side of the channel; at 100-fold higher concentrations on the external side, MTSET fails to alter the C519 conductance, even after a 20-min exposure (data not shown). A negatively charged reagent bearing a sulphonate group, MTSES, converts the C519 channel to a low-conductance E519-like phenotype, also in two steps. The wild-type K519 channel does not show any pore modification by MTS reagents under similar conditions. A comparison of the altered channels (Fig. 4*c*) further suggests that the small effect of side-chain volume observed in the electrostatic point-mutants also applies to the 'unnatural' residues arising from the MTS reagents. The side-chain produced by MTSEA is slightly larger than lysine, and the conductance of the resulting pore is about 15% less than that of K519; a further 30% drop in conductance is brought about by the bulky trimethylammonium group donated by MTSET.

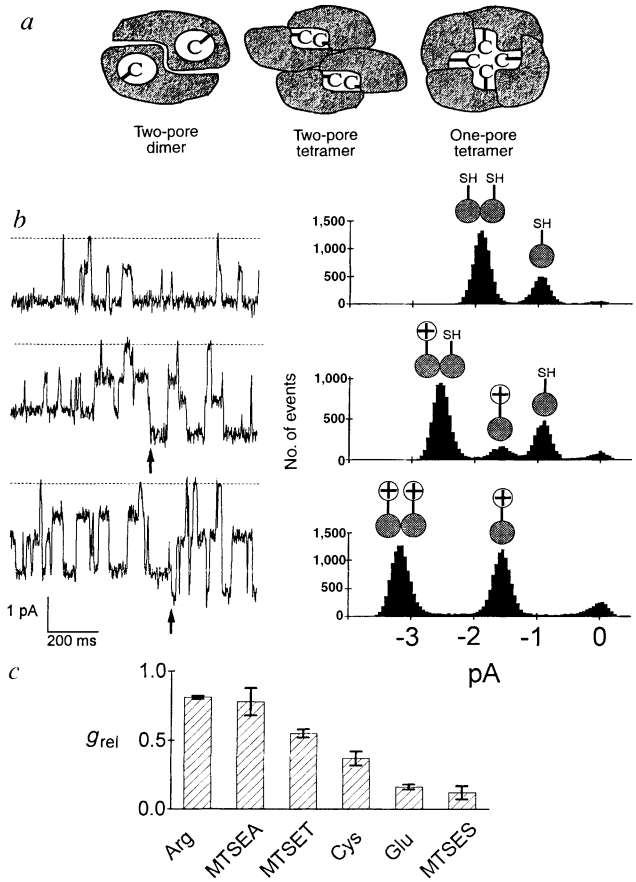

FIG. 4 Two-step reversion of C519 to K519-like channels by MTS reagents. *a*, Illustration of three alternative symmetrical subunit structures of the channel, with C519 drawn to indicate an influential position in the pore. *b*, Response of a C519 channel to 1 μM MTSEA. Channel records at −120 mV (left panel) before MTSEA reaction (upper trace), during the first MTSEA hit (arrow, middle trace), and during the second MTSEA hit (arrow, lower trace). Amplitude histograms (right panel) refer to traces before the first hit, after the first but before the second hit, and after the second hit, respectively; icons associated with histogram peaks represent the modification-state of the C519 side chains in open pores. *c*, Pore conductances, g_{rel}, normalized to that of wild-type ClC-0-K519, of C519 modified by MTS reagents and, for comparison, arginine, cysteine and glutamate substitutions.

From these experiments, we deduce two molecular features of the ClC-0 channel. First, the sidedness of MTS modification demonstrates that residue 519 faces the internal solution. The electrostatic influence of this residue suggests that it provides mechanistically relevant surface charge near the cytoplasmic entryway of the Cl^- conduction pathway. Second, these results provide strong evidence that the ClC-0 channel is a two-pore homodimer, as previously proposed on the basis of velocity-sedimentation behaviour in nondenaturing detergent[10]. Residue 519 exerts its graded electrostatic influence on permeation exactly twice in the channel complex, once separately in each conduction pathway. Moreover, the frequencies of the hybrid C519–K519 and homomeric C519 channels in our preparations are close to the 2 : 1 prediction for a random mixture of equally expressed subunits in a dimer.

The ClC-0 channel is thus constructed by a 2-pore–2-polypeptide architecture unprecedented among eukaryotic ion channels. This structural design is distinct from that of the familiar channels gated by voltage, cyclic nucleotides, or neurotransmitters, which are built around single pores formed on symmetry axes at the junction of 4 or 5 similar subunits[22]. In ClC-0, the twofold axis, assumed to exist in the homodimer, cannot be coincident with either pore. Instead (Fig. 4*a*), the two identical pores must be formed off-axis[10], as in the 3-pore–3-polypeptide construction of bacterial porins[23,24]. Consequently, we expect that many parts of the polypeptide contribute pore-determinants, in contrast to the localized pore-forming sequences of voltage-gated cation-conducting channels[25]. ClC-0 is the only member of the ClC family studied at the single-channel level[14], and so it is not known whether its double-barrelled structure is a unique feature of this homologue or a general property of the entire family. Because we view a membrane protein's quaternary architecture as a fundamental characteristic rather than a modulatable molecular embellishment, we speculate that the two-pore homodimeric character of ClC-0 applies to all members of this large family of Cl^- channels.

Note added in proof: Similar results were obtained by Ludewig *et al.* (*Nature*, this issue pp. 340–343). □

Methods

Purification, expression and reconstitution of ClC-0 channels. Functional ClC-0 channels were purified from *Torpedo californica* electroplax plasma membranes or from HEK-293 cells transiently transfected with a cDNA coding for ClC-0 from *Torpedo marmorata*[1]. CHAPS-solubilized channels were bound to an immunoaffinity resin based on the RM2 epitope (EGQQREGLEAVKVQTEDP, near residue 650), and eluted with the RM2 peptide as described[10]. Purified ClC-0 (5–10 μg) was reconstituted by gel filtration into proteoliposomes (2 mg ml^{-1}), which were subsequently fused into planar lipid bilayers, to give single-channel behaviour identical to that seen without CHAPS treatment[10]. The ClC-0 clone[1] (generously supplied by T. J. Jentsch) was altered by removal of the flanking untranslated sequences and by incorporation of convenient restriction sites, and transferred to the mammalian expression vector pCDNA3 (Invitrogen). HEK-293 cells (50 100-mm plates) were transfected overnight with 0.5 mg CaP_i-precipitated DNA, collected after 40 h, and extracted with CHAPS for subsequent purification of ClC-0. Hybrid channels were purified similarly after transfection of HEK-293 cells with equal amounts of ClC-0-C519 and of ClC-0-K519 cDNA in which the RM2 epitope had been replaced by the HA epitope.

Single ClC-0 channels were recorded under asymmetrical ionic conditions, with 5 mM Tris-Cl, pH 7.4, and 600 mM NaCl (internal), 200 mM NaCl (external). Records were filtered at 200 Hz and analysed as described[13]. We confined our analysis to properly oriented single channels displaying stationary gating among well defined conductance levels for enough time to collect data at a minimum of 5 voltages; these data-selection criteria applied to over 50% of all the single-channel incorporation events we observed.

Modification of single channels by MTS reagents. MTS derivatives[21] carried the following functional groups: ethylamino (MTSEA), ethyltrimethylamino (MTSET), ethylsulphonate (MTSES). After incorporation of a single C519 channel in a bilayer, a control *I–V* curve using at least 10 voltages was recorded. The desired methanethiosulphonate reagent (1–10 μM) was then added to the internal solution, with a 15-s burst of vigorous stirring, and data were continuously collected over the next few minutes at a constant holding voltage in the range −80 to −120 mV. Individual 'hits' by MTS reagents were observed as abrupt changes in single-channel current, as in similar experiments on diphtheria toxin channels[26].

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Two physically distinct pores in the dimeric ClC-0 chloride channel

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THE *Torpedo* chloride channel ClC-0 (ref. 1) is the prototype of a large family of chloride channels that have roles in transepithelial transport[2] and in regulating electrical excitability[3–6] and cell volume[7]. ClC-0 opens in bursts with two identical conductance levels of ~8 pS (refs 8–10). Hyperpolarization slowly increases the probability of bursts ('slow gating'), and depolarization increases channel opening within bursts ('fast gating'). Replacing serine 123 by threonine changes rectification, ion selectivity and gating, but retains the typical bursting behaviour with two identical independent albeit reduced, conductance states (~1.5 pS). Coexpression with wild-type ClC-0, either as covalently linked concatamers or as independent proteins, leads to bursting channels with two different pores. Our experiments strongly suggest that conductance, ion selectivity and 'fast' gating are determined only by the single subunit forming a single pore, independent from the attached pore; in contrast, 'slow' gating is a function of both subunits. Thus ClC-0 is a homodimer with two largely independent pores.

A region between transmembrane domains D2 and D3 is highly conserved across all known ClC proteins[11], including those from yeast[12] and bacteria[13] (Fig. 1). In ClC-0, a conservative substitution of serine 123 by threonine (S123T) led to fast gating being slowed (Fig. 2*a*) and a slight outward rectification of open channels and a relative loss of ion selectivity (Fig. 2*b*). Wild-type ClC-0 activates slowly at negative voltages owing to the voltage-dependent slow gate, but no such effect was observed with S123T up to −150 mV (Fig. 2*c*).